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Concordance of quantitative damage within the diencephalon and telencephalon following systemic pilocarpine (380 mg/kg) or lithium (3 mEq/kg)/pilocarpine (30 mg/kg) induced seizures

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Abstract

We tested the hypothesis that a single systemic injection of 380 mg/kg of the muscarinic agonist pilocarpine would produce more diffuse and severe seizure-induced brain damage than a single injection of lithium (3 mEq/kg) followed 4 h later by $< 1/10$ the dosage of pilocarpine. The hypothesis was not supported; the pattern of quantitative brain damage 50–60 days after the seizures were elicited by either treatment was comparable within the limits of measurement error. Within the diencephalon and subcortical telencephalon the same structures were either damaged in a similar quantitative manner or were spared. Only five of the 119 damaged structures exhibited statistically significant treatment differences at $P < 0.01$. The results are compatible with the explanation that lithium may enhance the excitotoxic effects of subsequent muscarinic stimulation.

Key words: Lithium; Pilocarpine; Seizure-induced damage; Diencephalon; Telencephalon; Rat brain damage

1. Introduction

A relatively large (e.g. 380 mg/kg) single, systemic (s.c.) injection of the muscarinic agent pilocarpine is usually required to elicit maintained limbic motor seizures [13]. However, Honchar et al. [3] found that only 30 mg/kg pilocarpine was required to elicit identical behaviors if the rats had been injected s.c., 24 h earlier, with only 3 mEq/kg lithium chloride. The seizures do not occur if the temporal order of the two drugs is reversed [10,11]. Neuropathological examination indicated that multiple regions within the cerebral cortices, diencephalon and telencephlon were damaged within 24 h after the seizure onset.

However, (1) similar behaviors do not prove comparable patterns of neuronal activation, and (2) qualitatively similar patterns of neuronal dropout with 48 h following the injection of either a single large bolus of pilocarpine or lithium and then pilocarpine [3] may not necessarily reflect the final quantitative damage that is evident 50–60 days later when the consequences of transneuronal degeneration and delayed mortality would be more evident [11]. The typical mortality of rats injected with either of these two treatments is reduced by injection of acepromazine (Atravet; Ayerst Labs, Montreal, Canada) within 30 min after seizure onset. Consequently, quantitative indicators of delayed and permanent brain damage can be determined by experimental design rather than by spontaneous mortality.

In several studies [2,4,6,10–12], a remarkably consistent pattern of damage for ~100 discriminable (as defined by Paxinos and Watson [5]) structures within the diecencephalon and telencephalon has been noted. The pattern of damage is conspicuous and clear by light microscopy at 100 and 400 × when the brains are fixed in ethanol-formalin-acetic acid or EFA [8]. Averaged estimates (means of several scores of damage along a 0 (none) to 10 (total) scale) of quantitative damage within each structure at 100 × are strongly correlated ($-0.78 < r < -0.92$) with the quantitative measures of neuronal density at 1000 × [6,9]. The validity and reliability of these estimates of quantitative damage are stable since correlation coefficients > 0.80 were found between these values (rated under blind

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Table 1
Means and S.D. values of percentage of area damaged within the cerebrum of rats 100 days after seizure induction by either 380 mg/kg pilocarpine or lithium (3 mEq/kg) and pilocarpine (30 mg/kg) (Paxinos and Watson abbreviations.)

Structure	Pilocarpine (380 mg/kg)		Lithium/Pilocarpine	
	Mean	S.D.	Mean	S.D.
ENTL	40	40	50	27
ENTI	33	41	39	28
ENTM	10	12	44	23 *
DSC	00	00	45	15
PAS	25	38	08	14
PRS	32	21	23	19
S	47	31	61	38
DG	51	33	10	25 *
CA1	88	12	86	17
CA2	95	08	95	31
CA3	78	25	83	31
CA4	51	31	27	30
IBI	25	41	00	00
OC1M	00	00	18	18
OC2L	33	08	64	15
OC2MM	66	16	81	14
OC2ML	08	20	11	20
RSG	61	20	48	29
RSA	41	27	19	20
CG2	58	41	17	21 *
CG1	42	34	05	12 *
CG3	00	00	00	00
PRH	58	31	51	31
TE2	16	41	00	00
TE1	05	12	22	40
TE3	05	12	20	40
PAR1	66	33	42	38
PAR2	65	38	41	44
FR2	21	31	10	17
FR1	20	33	18	23
HL	30	46	23	35
FL	33	51	27	41
FR3	00	00	32	46
GI	06	10	18	21
AI	48	34	68	37
CI	23	23	34	43
DI	00	00	21	33
APIR	43	35	59	33
PIR	80	22	95	18
DEN	73	23	81	31
VEN	93	12	97	06
SNC	00	00	00	00
SNR	100	00	97	09
SNL	00	00	00	00
CPU	28	24	42	11
ASTR	11	13	00	00 *
FSTR	00	00	18	06
EP	00	00	13	32
GP	10	16	06	15
VP	83	20	27	09
PMCO	78	13	86	18
PLCO	62	26	76	25
ACO	38	39	07	16
CXA	53	41	44	32
AHIPM	38	23	23	27
AHIPL	61	37	46	43
BLP	68	37	61	32
BMP	55	23	73	34

Table 1 (continued)

Structure	Pilocarpine		Lithium/Pilocarpine	
	Mean	S.D.	Mean	S.D.
BLA	48	33	49	24
BLV	83	25	80	36
BM	50	54	45	52
BMA	76	40	50	50
LADL	17	21	22	28
LAVM	78	24	73	27
LAVL	92	20	86	32
LA	33	51	27	46
MEPD	17	41	23	41
MEPV	50	45	45	52
ME	16	40	10	30
MEAD	00	00	13	03
MEAV	33	51	09	30
MEA	33	51	00	00
CEL	00	00	00	00
CEM	00	00	00	00
CELCN	00	00	00	00
CEMVP	00	00	00	00
CECL	00	00	00	00
CEMAD	00	00	00	00
I	00	00	00	00
IM	16	40	00	00
IMG	25	41	00	00
AAV	83	20	91	30
AASH	16	40	00	00
AAD	00	00	00	00
AA	00	00	00	00
MGD	46	37	46	42
MGV	25	41	21	31
MGM	38	37	26	33
SG	63	40	60	38
MG	66	51	31	46
PIL	30	40	33	47
RETH	00	00	00	00
POT	43	41	61	37
ETH	83	20	91	30
PLI	08	20	16	23
PO	75	22	83	24
ANG	16	41	10	30
LPMC	26	38	62	40
LPLC	42	49	63	39
LPMR	53	26	67	36
LPLR	41	37	83	24 *
LDVL	77	41	81	23
LDDM	75	41	100	00
IMA	00	00	05	15
STR	00	00	00	00
DLG	45	33	53	32
VLG(PC&MC)	00	00	00	00
IGL	00	00	00	00
INTG	00	00	09	30
SUBG	00	00	00	00
VLG/EML/MHB	00	00	00	00
LHBL	25	41	05	15
LHBM	16	48	09	30
VPM	57	39	19	32
VPL	65	28	40	35
VM	78	24	73	22
VL	68	25	47	36
RT	00	00	00	00
PVP	08	20	03	09
SPFPC	08	20	45	15

Table 1 (continued)

Structure	Pilocarpine		Lithium/Pilocarpine	
	Mean	S.D.	Mean	S.D.
PF	83	25	62	46
CL	50	45	45	35
CM	27	38	10	30
PC	30	40	27	27
PV	00	00	10	30
PVA	00	00	10	20
PT	50	44	59	49
POMN	00	00	09	30
RH	15	22	00	00
RE/VRE	100	00	100	00
G	66	40	53	42
IML/IMVC	00	00	00	00
MD	66	51	59	44
IMD	77	25	41	37
MDL	63	36	87	22
MDPL	16	40	00	00
MDC	68	24	73	41
MDM	76	40	91	30
IAM	83	40	18	40 *
AM	66	52	73	47
AD/AV	00	00	00	00
AVDM	33	51	27	47
AVVL	17	40	32	46
IAD	00	00	45	50

* $P < 0.01$ for both parametric and nonparametric tests.

conditions) for the mediodorsal thalamic complex and numbers of errors in the radial arm maze for rats in which lithium/pilocarpine seizures had been induced ~ 40 days before the behavioral measurements [2].

We hypothesized that quantitative indices of the multifocal brain injury that is evoked by either treatment would demonstrate significantly greater damage in the brains of rats that received a single systemic dosage of 380 mg/kg pilocarpine relative to rats that received 3 mEq/kg lithium first and then only 30 mg/kg pilocarpine, despite their similarities in seizure-related behaviors. If the hypothesis were not supported, then the likelihood that pretreatment with lithium simply enhanced the sensitivity of muscarinic receptors to lower dosages of pilocarpine would be suggested. Because rats that receive these or related concentrations of lithium and pilocarpine but do not seizure have never exhibited any evidence of brain damage, we have concluded that the protracted seizures evoke the necrosis [11,12].

2. Methods and materials

A total of 17 90–110-day-old Wistar albino male rats served as subjects. They constituted all of the animals: (1) that had received one of the two treatments; and (2) whose body weight ranged within the normal values for this age and sex. The rats had been obtained from Charles River Breeding (Quebec) and had been housed three per cage in standard wire housing within temperature controlled rooms; the L:D was 12:12 (light onset at 07:30).

11 of the rats received 3 mEq/kg lithium chloride (3 mEq/cc) s.c. followed 4 h later by 30 mg/kg pilocarpine. (The 4-h lithium–pilocarpine interval produces essentially the same effect as the 24-h interval [7,8].) Six of the rats received 380 mg/kg s.c. pilocarpine. Each rat was then placed in a single aquarium and observed. Seizure onset time (SOT), as defined by rearing and forelimb clonus, was measured to the nearest second. Within 1 min after SOT (~ 30–35 min after pilocarpine injection), the animals were injected s.c. behind the neck with 25 mg/kg acepromazine (Atravet). Rats from each of the two conditions were injected only once on 1 of 4 separate days.

After the acepromazine treatment the rats were returned to their homecage and given the usual postseizure procedure [11]. Within ~ 7–10 days, the rats appeared normal. Occasional spontaneous seizures were observed (estimated one overt display per day/rat); typically, the rat's ongoing behavior (e.g. eating), ceased and the following sequence was displayed: forepaw clonus, rearing on hindfeet and a fall backwards. Within 20–30 s, it would then exhibit an enhanced startle response but then returned to the preseizure behavior within about another 30 s.

~ 50–60 days after the injection (and initiation of the initial limbic seizures), the rats were decapitated and the brains were fixed in ethanol-formalin-acetic acid [4,11]; there is no difference in cytoarchitectural resolution or clarity even at 1000× power magnification between brains that have been perfused or not perfused with this technique [8,9]. After processing, 10-μm coronal sections were prepared by microtome and stained in toluidine blue O. All of the structures designated by Paxinos and Watson [5] were observed at 100 and 400×, every 200 μm, between the posterior commissure and anterior commissure. The percentage of each structure that contained neuronal dropout, gliosis and/or G factor (dense neuropil debris [4]) was determined for each of the two to four sections that contained a given structure; the proportion of cross-sectional area that contained any of these anomalies was estimated to the nearest 10% by an experienced histomorphologist (O. Peredery) who was not familiar with the experimental treatment of the animal. The average (mean) of these estimates for the two to four sections for each Paxinos and Watson structure was used as the inference of percentage of neuronal dropout.

Values for all structures within the commissural boundaries were loaded onto a VAX 4000 computer. One-way ANOVA and nonparametric (Kruskal–Wallis) ANOVA for verification were completed between the group that had received 380 mg/kg pilocarpine only and the group that had received the lithium/pilocarpine combination for all of the structures that exhibited damage. Regression analysis was completed between means for the percentages of neuronal dropout for each structure for the two groups (the structures served as 'cases'). The values for the rats that received the pilocarpine only constituted the dependent variable while values from the rats that received the lithium/pilocarpine were the predictors; standardized residuals, to discern which structures deviated by > 2 S.D. values from the expected values, were obtained. All analyses involved SPSS software. Because of the numbers of structures and the occasional heterogeneity of variance between groups, differences were accepted as statistically significant when they were $< P = 0.01$ according to both the parametric and nonparametric analyses.

3. Results and discussion

One-way ANOVA indicated no statistically significant difference ($F_{1,15} = 2.38$, $P < 0.05$) in SOT between

the rats that received the combination of lithium/pilocarpine and the single large dosage (380 mg/kg) of pilocarpine. The mean and S.D. values for the two groups were 33.6 (4.1) and 25.5 (17.0) min, respectively. However, Kruskal–Wallis analysis (because of the statistically significant heterogeneity of variance: Bartlett-Box = 12.92, $P < 0.001$) showed that the SOT of the 380 mg/kg rats was significantly faster ($\chi^2 = 4.66$, $P = 0.03$) than the rats that received only 30 mg/kg pilocarpine after the 3 mEq/kg lithium. Despite these marginal differences, the SOTs for both groups were well within 1 S.D. of the mean value for the ~500 male rats of comparable age that we have treated with 3 mEq/kg lithium and 30 mg/kg pilocarpine 4 h later [7,8]. Subsequent to recovery, the numbers of spontaneous overt seizures (e.g. forepaw clonus and rearing), were infrequent (estimated 1/day) and intermittent [11,12].

There was no statistically significant difference ($t = 0.83$, $P > 0.05$) between the mean overall brain damage for the rats that had received 380 mg/kg pilocarpine only and the rats that had received the combination of lithium/pilocarpine; the mean and S.D. values for the two groups were 39.9 (28.8)% and 38.6 (29.8)%, respectively. The mean and S.D. values for the percentage of cross-sectional cell loss for each structure that exhibited any damage are shown in Table 1. For emphasis some of the structures that did not show any damage are included. For example, none of the components of the central amygdaloid nucleus exhibited any damage and have never shown any evident necrosis following seizures that were induced by the lithium/pilocarpine injections. There has never been any obvious damage within the hypothalamus, most of the ventral striatum (e.g. nucleus accumbens), the rostral ventral forebrain (e.g. Islands of Calleja) or the midbrain, except for the substantia nigra reticulata.

Of the 119 structures that displayed any discernable damage, only five structures exhibited significance as defined by preestablished criteria. The results of the ANOVAs indicated that the rats that received only the pilocarpine (380 mg/kg) exhibited significantly ($P < 0.01$) more damage within the first region of the cingulate gyrus, the dentate gyrus, the amygdaloidstriatal transition and within the interanteromedial thalamus. The lithium/pilocarpine group exhibited significantly more damage within the medial entorhinal cortices relative to the pilocarpine only group.

Regression analysis demonstrated a correlation of 0.86 ($F_{1,174} = 485.17$, $P < 0.001$) (note: 57 of the ~90 additional structures that were rated did not display discernable damage, i.e., 0 values, in either group were included in order to fairly represent those structures without overly biasing the analysis with multiple cases of pairs of 0 values) between the two treatments for percentage of damage within the structures that exhibited any discernable necrosis. When only the 119 damaged structures were included, the r value was 0.80 ($F = 218.30$). The structures that exceeded two standardized residuals were similar for both analyses. Relative to the rats that received the lithium/pilocarpine, the rats that received the single large dosage of pilocarpine exhibited more cell loss within the interanterodorsal thalamus, cingulate gyrus, dentate gyrus, intermediodorsal thalamic nucleus, ventral posteriomedial thalamic nucleus and medial geniculate (proper) nucleus. On the other hand, the intramedullary thalamic area and laterorostral part of the lateral posterior nucleus of the thalamus exhibited less damage in the pilocarpine only rats than would be expected from the lithium/pilocarpine treatment only. Interestingly, the interanterodorsal and medial geniculate (proper) structures are among the smallest (< 250 mm^3) of the thalamic nuclei and would be most prone to measurement error.

The results do not support the hypothesis that a single systemic injection of 380 mg/kg pilocarpine evokes more severe, long-term and permanent neuronal loss than a single injection of lithium followed 4 h later by $< 1/10$ that amount of pilocarpine. The bivariate correlation between damage scores for the two treatments ranged from 0.80 to 0.86; structures that exhibited the greatest discrepancies were also those most prone to measurement error because of their small size. Only five of the 119 structures that exhibited any discernable damage also demonstrated treatment differences. At least one and possibly two of these differences would be expected by chance alone ($P = 0.01$) to show group differences. If the structures that were evaluated but did not display any damage (although potentially they could have exhibited group differences) are included, then significant group differences existed in $< 1\%$ of the structures in this portion of the rat brain.

The absence of large and significant differences in the general pattern of quantitative damage between the two treatments could suggest that the biochemical consequences of a systemic preinjection of relatively low concentrations of lithium followed by a subseizure dosage of pilocarpine simulates the effects of injecting $> 10\times$ the amount of this muscarinic agent. One possibility is that preinjection with lithium strongly enhances the binding capacity of pilocarpine to muscarinic receptors. The 100-fold increase in the concentration of a metabolic product of the important membrane (receptor) component phosphatidylinositol [3] is compatible with but not exclusively supportive of this conclusion. Involvement of muscarinic (M1 and M2 subtypes) receptors in this spatial pattern of damage within the thalamus is also suggested by the sparing of the anterior group and the reticular nucleus since they exhibit minimal reactivity to M35 antibody [1].

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